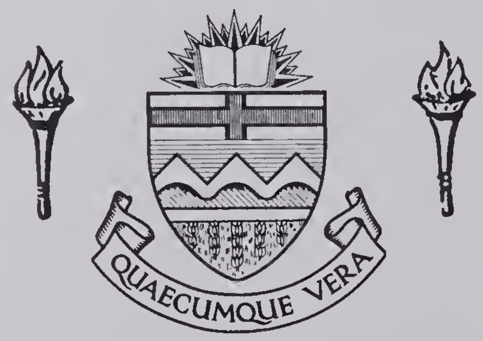
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THE UNIVERSITY OF ALBERTA

CULTURE STUDIES AND MATING REACTIONS IN *CYATHUS HELENAE* BRODIE AND RELATED SPECIES

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "CULTURE STUDIES AND MATING REACTIONS IN *CYATHUS HELENAE* BRODIE AND RELATED SPECIES", submitted by Alexander Olchowecki in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Cyathus helenae Brodie, a new species of the Nidulariaceae from the Canadian Rockies, has been investigated and was found to be heterothallic and to have the so-called "tetrapolar" type of sexuality. On the basis of pairing reactions, the species is probably closely related to *C. striatus* (Huds.) ex Pers. The two species differ markedly in their cultural characteristics.

Monosporous cultures of *C. helenae* are extremely variable and phenomena observed in these cultures resemble phenomena previously reported to be exclusively associated with heterokaryotic reactions in tetrapolar species of other Basidiomycetes.

Both haploid and diploid cultures of *C. helenae* produce a bacteristatic substance that is heat-stable and appears to stimulate bacterial growth at low concentrations but inhibits bacterial growth at higher concentrations. Some other species of *Cyathus* tested also produce metabolites with similar properties.

ACKNOWLEDGEMENT

The writer is indebted to Dr. Harold J. Brodie for his encouragement and guidance; his criticism of the manuscript is also acknowledged. This work is made possible through the use of equipment provided by grants to Dr. Brodie by the National Research Council of Canada, Ottawa, Canada.

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INTRODUCTION

A pattern of sexuality in fungi involving multiple incompatibility alleles at two loci was first described in 1920 by Kniep (14) in the hymenomycetous fungus *Schizophyllum commune* Fr. Basidiospores, and subsequently mycelia, of four distinct types are produced by each 'tetrapolar' fruit-body. Kniep interpreted this type of sexual manifestation as the result of the occurrence of sex-determining factors at two genetic loci; e.g.

Zygote		Spore Progeny
AaBb	-----Meiosis-----	AB, Ab, aB, ab

In all possible crosses among the four types of mycelia, matings occur only in those combinations which restore the double heterozygote; namely, AB x ab, and Ab x aB.

Extensive subsequent investigations have resulted in the rapid accumulation of a voluminous literature on the sexual process in the hymenomycetous fungi. However, little purpose would be served by a review of the literature in the present paper as this has been done in detail by Whitehouse (21, 22) and Raper (18).

Cyathus, a genus of the Gasteromycetes, has been studied extensively by Brodie (4, 5). All species within the genus have been shown to be heterothallic and tetrapolar,

as are most other members of the family Nidulariaceae to which *Cyathus* belongs.

Cyathus helenae Brodie, a species recently described from the Canadian Rockies (6), is the central subject of the present investigation. Monospore cultures of this species had not been obtained previously and it seemed desirable to analyze the sexuality pattern of the new species for purposes of comparison with other possibly related members of the genus. Because of its unusual (and probably unique) habitat, an examination of the behaviour of the latter fungus in culture was an added attraction for study [see Brodie (6)].

Cyathus helenae was recognized as a distinct species on the basis of clear-cut morphological characteristics and apparently unique ecological requirements. However, in his discussion, Brodie (6) recognized that it appears to be more closely related to *C. striatus* (Huds.) Willd. ex Pers. than to any other species of *Cyathus*. The second purpose of the present investigation was, therefore, to find out whether or not any relationship between these two species exists, in terms of a genetic relationship as might be evidenced by attempts to cross the two species, and in terms of similarities or differences that might appear between the species in their behaviour in culture.

The accidental discovery that *C. helenae* produces a bacteristatic substance, late in the course of the research reported herein, prompted preliminary investigation into the action of the substance.

MATERIALS AND METHODS

Origin and Data Concerning Specimens Used

Two strains of *Cyathus helenae* and two strains of *C. striatus* were investigated. The following specimens of mature fruit-bodies were obtained from the Herbarium of Dr. H.J. Brodie (the numbers cited are those of the Brodie Herbarium):

No. 1500, *C. helenae*, Mountain Park, Alberta, August 20, 1965, H.J. Brodie

No. 67016, *C. helenae*, Drumheller, Alberta, June 8, 1967, H.J. Brodie

No. 66145, *C. striatus*, 10 mi. south of Mosely, Alberta, October 5, 1963, C.D. Bird (No. 9090)

No. 66146, *C. striatus*, Shaganappi Hill, SW Calgary, Alberta, July 28, 1963, A.A. Tennant.

Technique for Obtaining Monosporous Cultures

Peridioles removed from the basidiocarps were sterilized in a 0.5% solution of mercuric chloride for two minutes. After 3 subsequent washes (2 minutes each) in sterile distilled water, each peridiole was placed in a Petri dish containing approximately 10 ml. of sterile distilled water and cut into minute fragments to allow the basidiospores to be suspended separately in the water. The suspension was then poured on to a plate containing a

1/5-Normal concentration of Sucrose-Peptone Agar (Sucrose 2 gm., Peptone 1 gm., KH_2PO_4 0.4 gm., $MgSO_4$ 0.2 gm., Agar 15 gm., and water to make 1000 ml.). This suspension was then poured on to another Petri dish containing the same medium. Examination of the first plate under the low power of a microscope showed that a satisfactory number of spores remained on the surface of the medium, after the water suspension of spores had been decanted. The process was repeated until twenty Sucrose-Peptone-Agar plates had been similarly inoculated.

The covered Petri dishes were then placed in enamelware pans, covered with Parafilm to prevent desiccation, and incubated at 37°C. After 48 hours, the pans were removed to room temperature (22°C) and the Parafilm removed to allow the inoculated surfaces of the agar to dry.

The surfaces of the inoculated plates were scanned by placing each plate in an inverted position on the stage of a microscope and viewing the spores, through the agar, with a 32 mm. objective (5X) and a 10X eyepiece. The positions of germinating spores were noted by placing a small dot on the bottom of the plate with a felt marking-pen, in the optical path between the germinating spore and the objective of the microscope. The plates were then opened and small blocks of agar were removed from the noted locations with a sterilized scalpel. The blocks were trans-

ferred to Petri plates containing Brodie Medium Agar (4) and observed under the microscope. The pH of this medium, autoclaved, is 6.4. The blocks were trimmed, if required, to remove other germinating or ungerminated spores.

The mating type of each monosporous culture was determined by pairing each culture with all of the others. This was done by placing inocula of the desired cultures side by side on Brodie Medium Agar (abbreviated to BMA in subsequent pages) in Petri plates. Following a 5-6 day incubation period at 22°C, the plates were inspected under the microscope in the manner described above for germinating spores.

The presence of clamp-connections at septa and at branch axils was the criterion adopted as an index of sexual compatibility (Fig. 1). Final confirmation of true clamp -connections[1] was made by mounting a small bit of mycelium on a slide and viewing it under a 40X objective of a phase contrast microscope (Fig. 3). Figs. 2 and 4 show the appearance of the clamp-connection-lacking haploid mycelium under similar magnifications.

All monosporous cultures, the mating type of which had been determined, were maintained as stock cultures on

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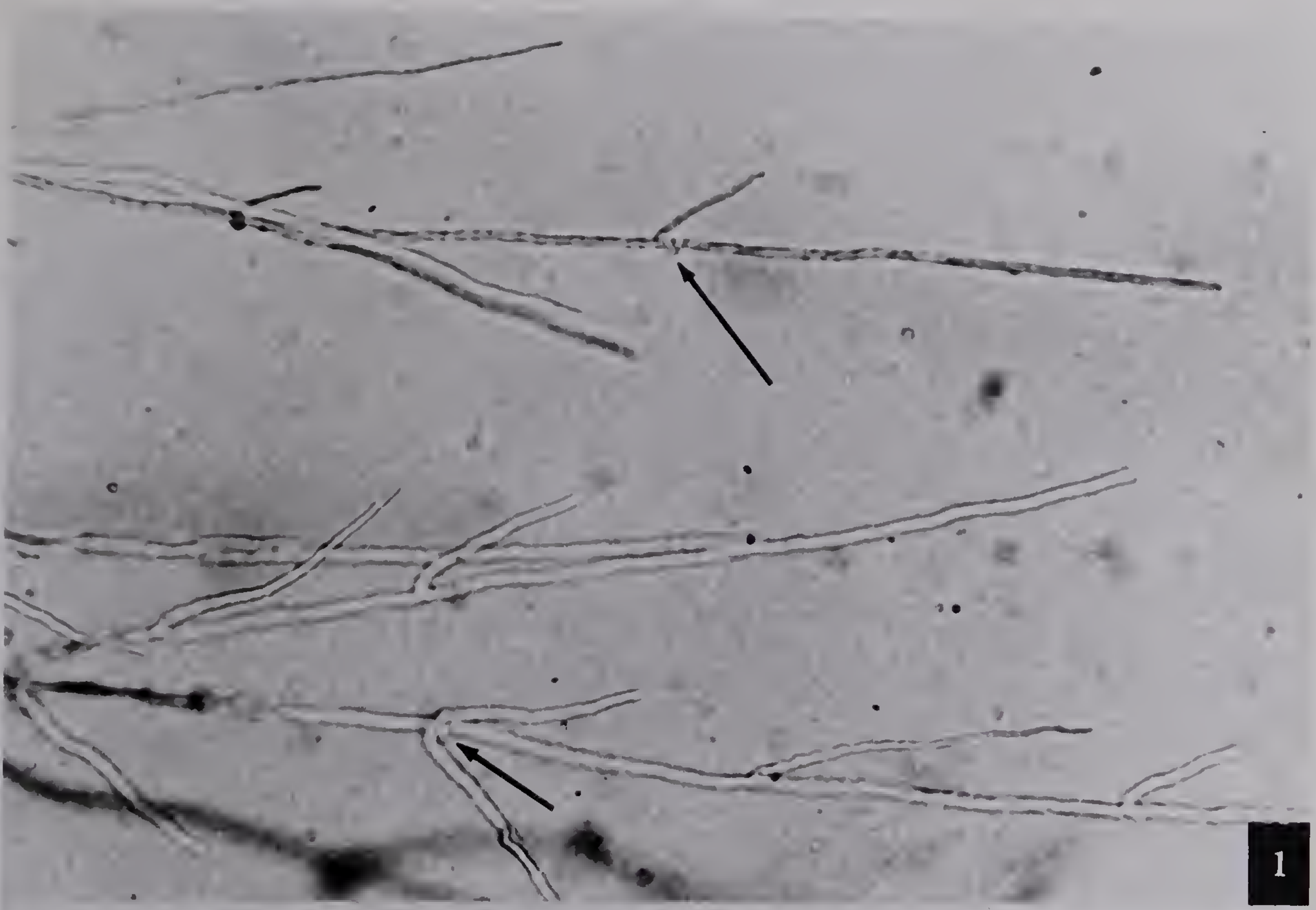
[1] "False clamp-connections" are of common occurrence in certain haploid mycelium pairings in hymenomycetes [see Raper (18)]. Caution is necessary to be sure that what is observed in mated mycelia is a true or a false clamp -connection.

PLATE I -- Haploid and Diploid Cultures
(*Cyathus helenae*)

Fig. 1. Diploid mycelium of *C. helenae* No. 1500 growing on Brodie Medium Agar (X250). Arrows point to clamp-connections, visible even at low magnification.

Fig. 2. Monosporous culture (haploid) of *C. helenae* (Culture No. 30) growing on Brodie Medium Agar (X250).



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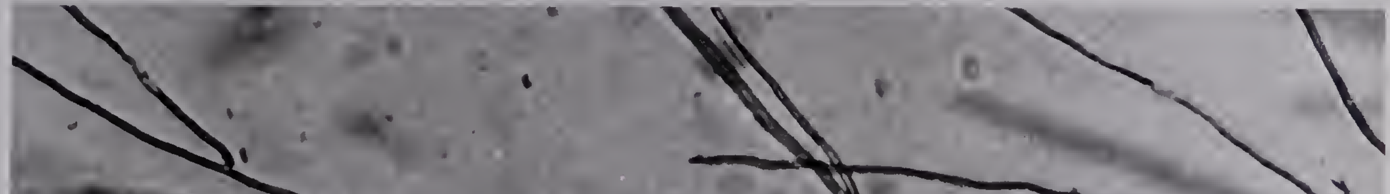

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PLATE I

PLATE II -- Diploid and Haploid Mycelia

(*Cyathus helenae*)

Fig. 3. Diploid mycelium of *C. helenae* showing clamp -connections at a septum and at a branch axil (X1600).

Fig. 4. Haploid mycelium of *C. helenae* (X1600). Note absence of clamp-connections at septa.

3

4

PLATE II

BMA slants at 15°C. Subcultures were made every 3-4 weeks on to BMA plates for observation of colour, growth rate, and other characteristics.

Methods for Detecting Bacteristatic Activity

Two methods were used to detect the activity of a bacteristatic substance observed to be produced by the fungus cultures. One was to cut 5 mm-square blocks from the fungus as it was growing on BMA plates and to place these blocks on Nutrient Agar plates seeded with a suitable dilution of *Staphylococcus aureus* Rosenbach. Suitable dilution was determined by the usual serial-dilution methods.

A second method involved growing the fungi in 500 ml. Erlenmeyer flasks containing 100 ml. Brodie Medium Liquid (Brodie Medium, less agar), at 5 times the 'normal' concentration of all ingredients per liter of solution. After 28 days' growth, the medium was passed through a sterile filter and the filtrate was mixed with an equal part of 3% agar solution in water. This was then poured into Petri plates and allowed to solidify. Blocks were cut from these plates and used in the same manner as were the living mycelia, as described above.

The methods described were also used to test activity against the following micro-organisms: *Proteus morganii* (Winslow *et al.*) Rauss, *P. rettgeri* (Hadley *et al.*)

Rustigian and Stuart, *Escherichia coli* (Migula) Castellani and Chalmers, *Aerobacter aerogenes* (Kruse) Beijerinck, *Bacillus subtilis* Cohn emend Prazmowski, *Streptococcus pyogenes* Rosenbach, *Sarcina lutea* Schroeter, *Micrococcus aurantiacus* (Schroeter) Cohn., *M. candidus* Cohn., *M. cyaneus* (Schroeter) Cohn., *M. conglomeratus* Migula, *M. denitrificans* Beijerinck, *M. flavis* Trevisan, *M. freudenreichii* Guillebeau, *M. luteus* (Schroeter) Cohn., *M. roseus* Flügge, *M. varians* Migula, *Parcolon* sp., *Serratia* sp. (pigmented), and *Serratia* sp. (non-pigmented).

Microscopy and Photography

All visual observations, as well as photomicrographs, were made using a Vickers' Phase-Contrast Microscope. Macrophotography was done using an Asahi Pentax SV camera fitted with a 55 mm. lens. The film used (throughout) was Kodak Panatomic-X, developed in Kodak Microdol-X developer. All photographs were printed on GAF VeeCee Rapid-A Paper and developed in 'Vividol'.

RESULTS AND OBSERVATIONS

Mating Reactions of Haploid Mycelia of *Cyathus helenae* and Related Species

Sixty vigorous monosporous cultures of *C. helenae* (No. 1500) were obtained as the result of four separate attempts to germinate spores, all from the same fruit-body. The basidiospores usually germinated in 7 days, and single -spore isolations were made over a period of 7-10 days. On the basis of their pairing reactions (Table I), the cultures were assigned to four mating types. Mating types AB and ab represent a compatible pair; similarly, Ab and aB represent the other compatible pair. All the haploid cultures of this strain of *C. helenae* were labelled H_1, H_2, etc.

Twenty haploid cultures of *C. striatus* (No. 66145) and eighteen cultures of *C. striatus* (No. 66146) were also obtained. The mating types of all of these were determined, and the results are presented in Tables II and III. The prefix "S" is used to denote cultures of *C. striatus* (No. 66145) and the prefix "T" is used to denote cultures of *C. striatus* (No. 66146). The latter is the tall form frequently found in mountain regions of Western Canada.

Each of the 60 haploid cultures of *C. helenae* was paired with two representatives from each mating type of *C. striatus* (No. 66145). The results of this operation are presented in Table IV. For simplification, only four

PLATE III -- Distribution of Haploid Mycelia (*Cyathus helenae* and *C. striatus*)

Table I. Distribution of Sixty Haploid Mycelia of *C. helenae* No. 1500 with Regard to Mating Type.

Table II. Distribution of Twenty Haploid Mycelia of *C. striatus* No. 66145 with Regard to Mating Type.

Table III. Distribution of Seventeen Haploid Mycelia of *C. striatus* No. 66146 with Regard to Mating Type.

Note: Mating type designation (AB, ab etc.) identify incompatibility factors for cultures listed in one table only and are not necessarily identical to similar designations in other tables (see Plate XII for revised designations).

Table I

Mating type	Culture number
AB:	30
ab:	1,2,3,4,5,6,7,11,17,18,19, 20,21,22,23,24,25,26,28,31, 33,35,36,37,38,40,41,42,43, 45,46,48,49,51,53,54,55,57, 58,59
Ab:	15,34,44,50
aB:	8,9,10,12,13,14,16,27,29, 32,39,47,52,56,60

Table II

Mating type	Culture number
AB:	6,19
ab:	1,2,4,8,9,10,11,12,17
Ab:	5,13
aB:	3,7,14,15,16,18,20

Table III

Mating type	Culture number
AB:	1,2,4,5,7,12,13,14,15,16
ab:	9,10,11
Ab:	6,8
aB:	17,18

PLATE III

PLATE IV -- Mating Reactions in *Cyathus*

Table IV. Results of Pairing Haploid Mycelia of *C. helenae* No. 1500 (H_{31} etc.) with Haploid Mycelia of *C. striatus* No. 66145 (S_6 etc.).

Table V. Results of Pairing Haploid Mycelia of *C. striatus* No. 66146 (T_{10} etc.) with Haploid Mycelia of *C. striatus* No. 66145 (S_6 etc.).

Table VI. Results of Pairing Haploid Mycelia of *C. helenae* No. 1500 (H_{31} etc.) with Haploid Mycelia of *C. striatus* No. 66146 (T_{10} etc.).

Table IV

	H_{30}	H_{31}	H_{34}	H_{32}
$S_6(\underline{AB})$		+		+
$S_4(\underline{ab})$	+		+	
$S_{13}(\underline{Ab})$		+		+
$S_7(\underline{aB})$	+		+	

Table V

	T_{13}	T_{10}	T_8	T_{17}
$S_6(\underline{AB})$		+		
$S_4(\underline{ab})$	+			+
$S_{13}(\underline{Ab})$				+
$S_7(\underline{aB})$		+	+	

Table VI

	T_{13}	T_{10}	T_8	T_{17}
H_{30}		+		+
H_{31}	+	+	+	+
H_{34}		+		+
H_{32}	+	+	+	+

PLATE IV

cultures of *C. helenae* and four of *C. striatus*, each representing one of the four mating types, are included in the table. A "+" sign indicates the development of mycelium bearing clamp-connections.

Crosses were carried out between the same cultures of *C. helenae* and *C. striatus* (No. 66146), as well as between the two strains of *C. striatus* (Nos. 66145 and 66146). The results of these crosses are presented in Tables V and VI, respectively.

Towards the end of this investigation, another strain of *C. helenae* (No. 67016 from Drumheller, Alberta) was obtained. Only ten monospore cultures, representing only two mating types, were obtained from these fruit-bodies. These haploid cultures corresponded to the mating types Ab and aB of *C. helenae* No. 1500 in all the above reported pairing reactions.

Finally, experiments were conducted to observe a related phenomenon, viz. that of nuclear migration [see Brodie (4), Fulton (12), Raper (18), Day (9) and Snider (19)]. To observe the effects resulting from the direction of nuclear migration, inocula were placed about 30 mm. apart on agar plates. The reaction at the juncture, where the two cultures met, was noted. Diploidization of a culture could be readily seen by the appearance of the diploid mycelium at the outer circumference of the culture, and could easily be confirmed microscopically. In the

event that unilateral migration of nuclei occurred, only one of the two mycelia was diploidized. The results of these experiments are presented in Plate V.

Attempts to Induce Fruiting of Hybrid Diploid Mycelia

Diploid mycelia, resulting from the various pairings between *Cyathus helenae* and *C. striatus*, were maintained on agar plates for lengthy periods of time, in no case, however, was fruiting observed.

A method of obtaining fruit-bodies described by Brodie (5) was also tried. Diploid mycelia were grown on wood-shavings moistened with Brodie Medium liquid. When these cultures were 6 weeks old they were transferred to five-inch earthenware pots, the mycelia being covered with an inch of sterile loamy soil. The pots were then placed on a bench in the greenhouse. No fruting resulted in any of twenty-five of such preparations.

In the Spring and Summer of 1967, forty more diploid cultures were prepared. In these later experiments forest litter was used instead of wood-shavings. Of these cultures, twenty were planted outdoors at various locations near the laboratory, and twenty were planted in shaded and exposed sites at the University of Alberta Botanical Garden. At the time of writing, no fruiting had occurred in any of the cultures planted under 'natural conditions'.

PLATE V -- Direction of Nuclear Migration

(*Cyathus helenae* and *C. striatus*)

Note: Arrows indicate presumed direction of nuclear migration. Double arrows indicate reciprocal exchange of nuclei.

Cyathus helenae

H_{31}(*ab'*) $\longleftarrow$ H_{30}(*AB'*)

H_{34}(*Ab'*) $\longleftrightarrow$ H_{32}(*aB'*)

Cyathus striatus

S_6 (*AB*) $\longrightarrow$ S_4 (*ab*)

S_7 (*aB*) $\longleftrightarrow$ S_{13}(*Ab*)

S_3 (*aB*) $\overset{\text{"Barrage"}}{\rule{4em}{0.4pt}}$ S_5 (*Ab*)

C. helenae X *C. striatus*

H_{31}(*ab'*) $\longleftarrow$ S_6 (*AB*)

H_{32}(*aB'*) $\longleftrightarrow$ S_6 (*AB*)

H_{31}(*ab'*) $\longleftarrow$ S_7 (*aB*)

H_{32}(*aB'*) $\longleftarrow$ S_7 (*aB*)

H_{30}(*AB'*) $\longrightarrow$ S_4 (*ab*)

H_{34}(*Ab'*) $\longleftrightarrow$ S_4 (*ab*)

H_{30}(*AB'*) $\rule{2em}{0.4pt}\ ?\ \rule{2em}{0.4pt}$ S_{13}(*Ab*)

H_{34}(*Ab'*) $\longleftarrow$ S_{13}(*Ab*)

PLATE V

Sectoring and Other Variations in Haploid Mycelia

A large proportion of the haploid cultures of *C. helenae* showed a tendency to produce variants which differed from the parent mycelium. Many of these variants retained their altered characteristics upon being subcultured, whereas others reverted to the parent type.

The most commonly observed type of variation was one in which the 'saltant'[1] had a growth rate equal to, or higher than, that of the parent. Of the 60 cultures studied, 21 gave rise to variants which appeared as outgrowths on the periphery of a culture and were distinguished macroscopically by dense mycelium closely appressed to the medium (Figs. 7 and 8). Observation under the microscope (Fig. 5) revealed that the hyphae of the variant grew deeply into the medium, had a characteristic "wavy" type of growth, and that the angle of branching differed markedly from that of the parent (Fig. 4). Other saltants were similar microscopically, but differed macroscopically in that they produced sparse aerial mycelium (Figs. 9 and 10). A characteristic of these saltants is that they acted as donors of nuclei in mating reactions, and that they themselves became diploidized very slowly, if at all.

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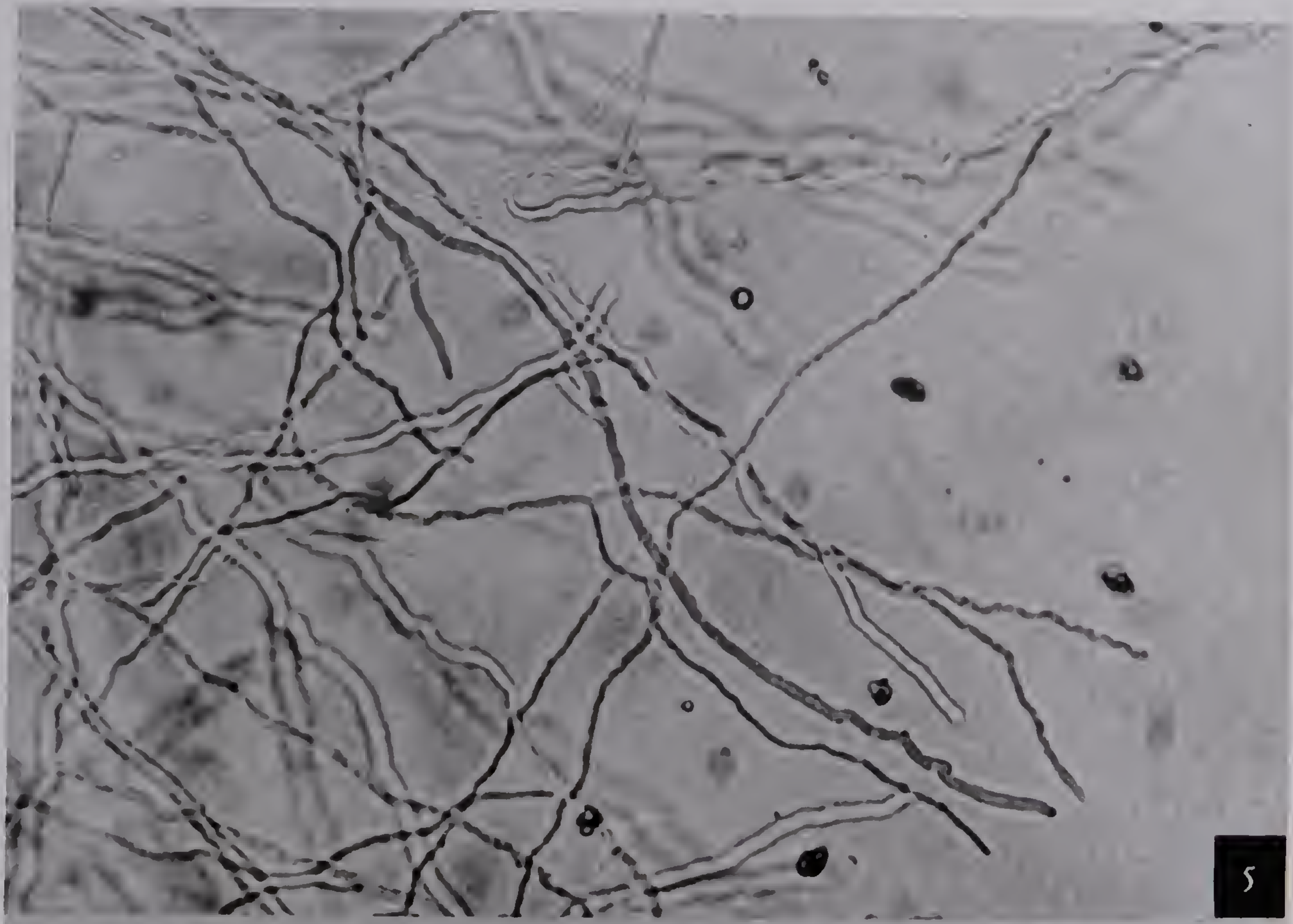
[1] In the present studies the terms 'saltant' and 'saltation' are used as defined by Butler and Jones (7); i.e., referring to a variation whose genetic basis has not been determined.

PLATE VI -- Variants of Haploid Mycelia

(*Cyathus helenae*)

Fig. 5. Mycelium of 'flat' saltant of *C. helenae* (X250) Note wavy type of growth and angle of branching (*cf.* Fig. 2).

Fig. 6. Mycelium of 'streak' saltant of *C. helenae* (X250). Note "ropy" appearance and proliferation of branches at intervals along a lead hypha.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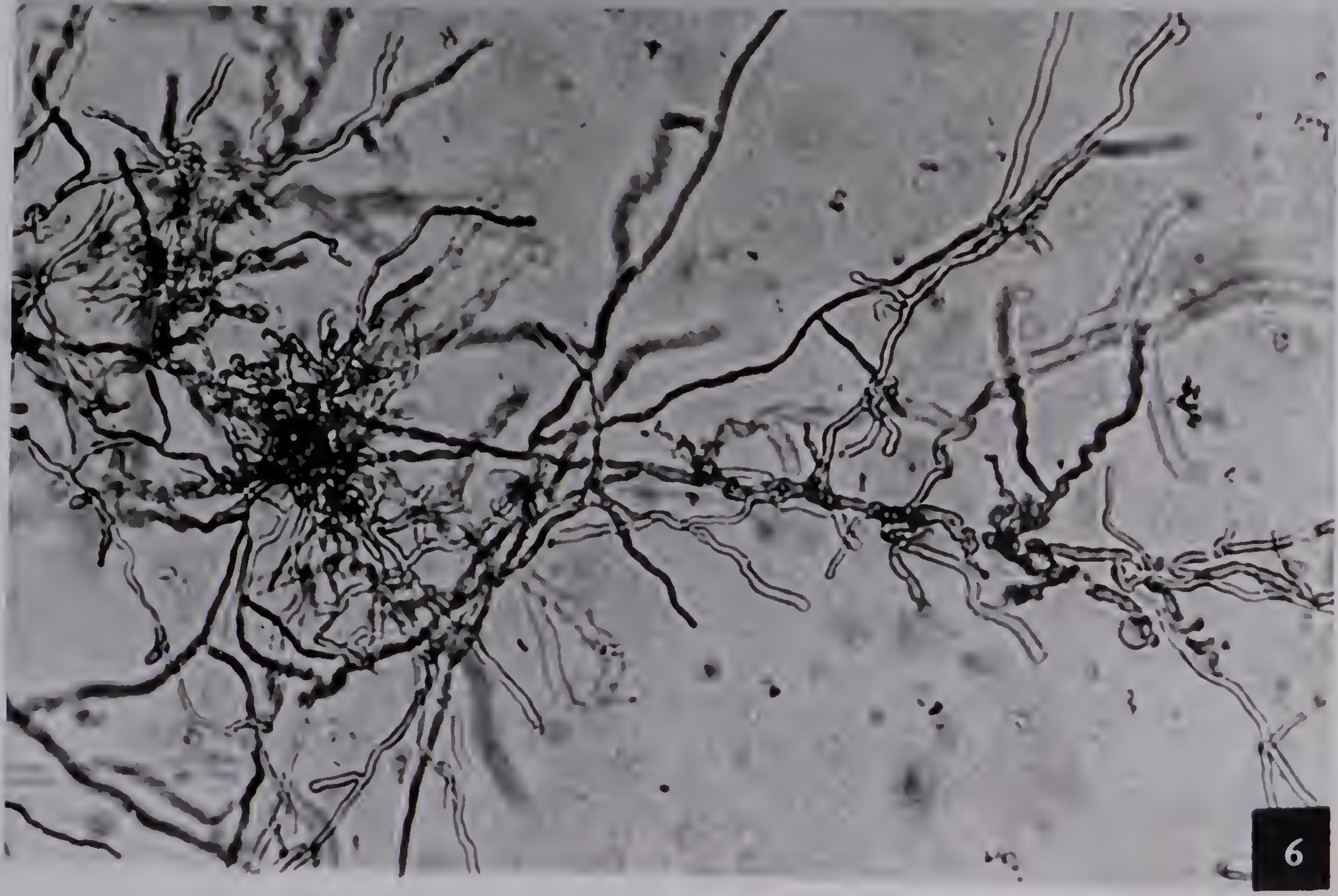
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PLATE VI

PLATE VII -- Variations in Haploid Cultures

(*Cyathus helenae*)

Fig. 7.	'Flat' variant of haploid culture of *C. helenae* (X1.5). Arrow indicates developing 'flat' mycelium.
Fig. 8.	Subculture of variant shown in Fig. 7. Note dense mycelium closely appressed to the medium.
Figs. 9 and 10.	'Flat' saltants (at the top of photograph) with sparse aerial mycelium.
Figs. 11 and 12.	'Streak' mycelium in haploid cultures of *C. helenae*. Arrows indicate 'streak' hyphae growing deep in the medium.

Note: Figs. 8 - 12 are 3/4 actual size.


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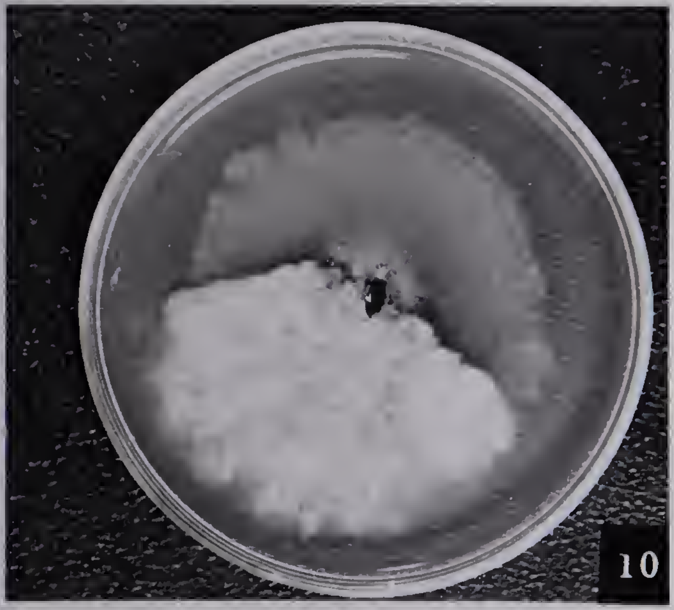
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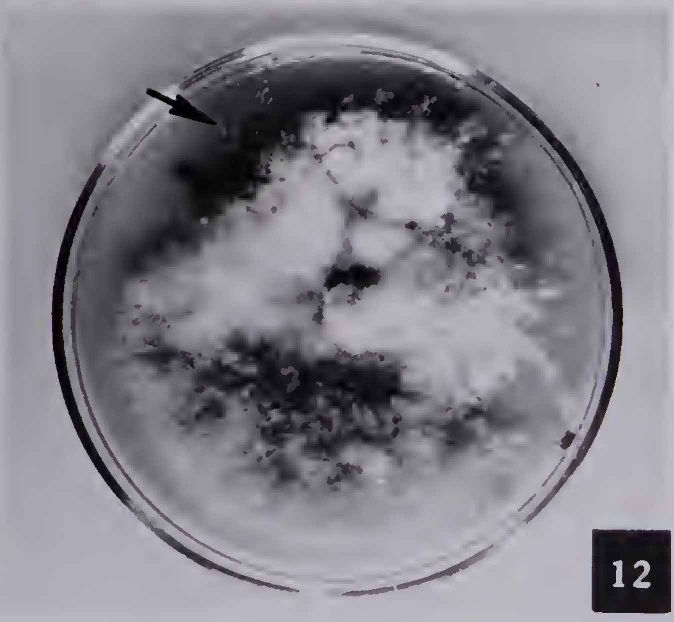
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PLATE VII

'Streak' mycelium similar to that described by Papazian (15) in *Schizophyllum commune*, was observed in four cultures (Figs. 6, 11 and 12). The 'streak' hyphae were entirely submerged in the medium, and aerial hyphae appeared only at some distance back from the growing edge. Upon being subcultured, 'streak' mycelium often gave rise to 'normal-looking' fluffy aerial mycelium. Such cultures, however, persisted in giving rise to 'streaks' after one or two weeks of growth.

Four types of sectors were observed. Two of these resembled the "A" and "B" types described by Pontecorvo (16). The "A" type (Fig. 13) reverted to the parent form upon being subcultured. There was only one occurrence of the "B" type, and no photograph is available. However, this was a pure white sector of a buff-coloured mycelium (Culture No. 30) and it has been maintained as a vigorous stock which has remained stable after many subculturings.

Two other types of sectors occurred, namely, "C" type (Fig. 14) and "D" type (Fig. 15) which, to the author's knowledge, have not been reported elsewhere. Subcultures from the "C" type reverted to the parent form which either did or did not sector further (Figs. 17 and 18). Mycelium from the "D" type exhibited very poor growth when transferred to fresh medium. Figure 16 shows such a subculture after 30 days growth.

PLATE VIII -- Sectors in Haploid Mycelium
(*Cyathus helenae*)

Fig. 13. "A" type sector in *C. helenae*. The saltant is characterized by sparse mycelium.

Fig. 14. "C" type sector. The saltant appears to have a slower growth rate and is a light buff -colour, compared to the pure white of the parent mycelium.

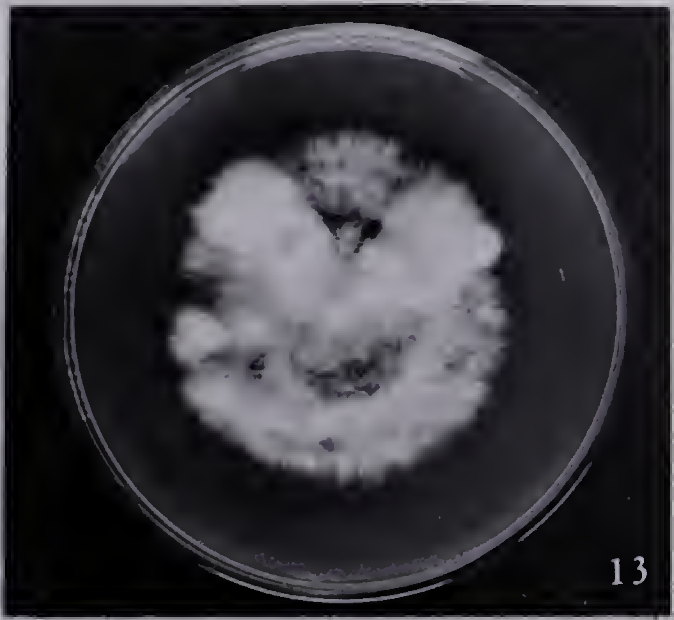
Fig. 15. "D" type sector. Note definite apex and dark 'interaction' zone (arrow).

Fig. 16. Subculture of mycelium from sector shown in Fig. 15 after 30 days growth.

Fig. 17. Subculture of mycelium from sector shown in Fig. 14. Note diffuse apex (arrow).

Fig. 18. Subculture of mycelium from sector shown in Fig. 14. Note absence of sectors.

Note: All figures approximately 3/4 actual size.



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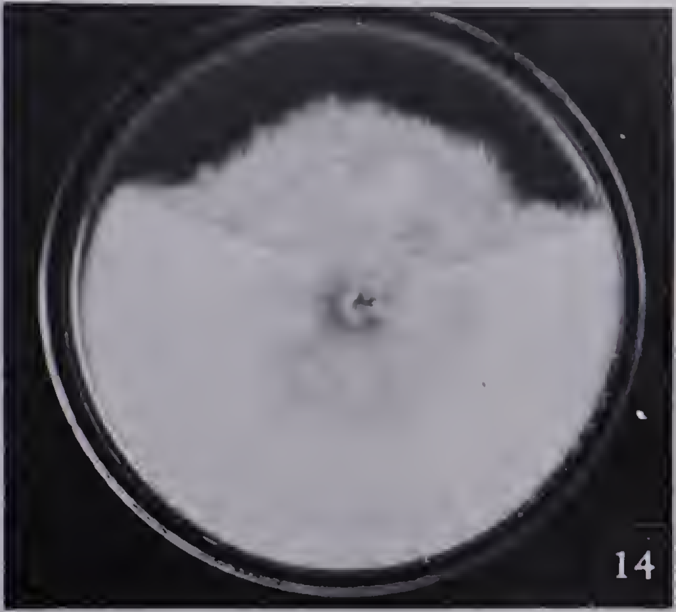

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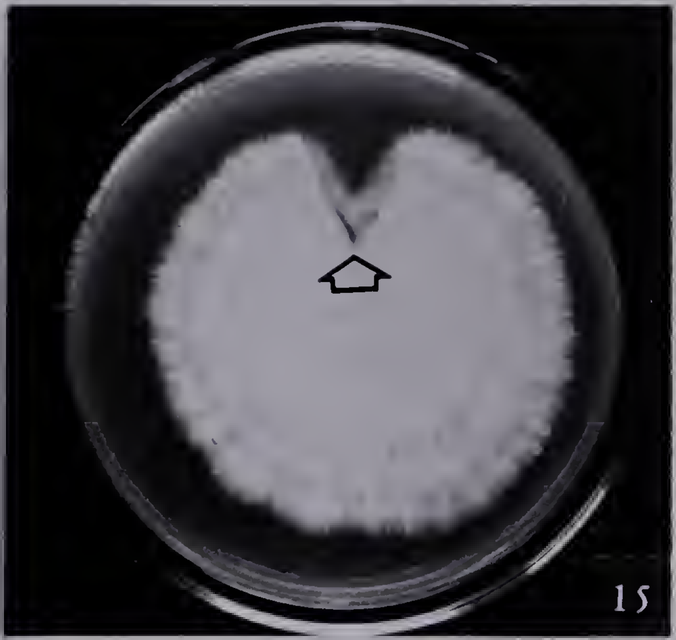

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PLATE VIII

Another type of variation is shown in Figs. 19 and 20. In this type, the saltant was dark brown in colour in contrast to the white parent culture. The angle of branching resembled that of a dikaryon, and false clamp-connections were present.

Other examples of variation are shown in Figs. 21 22, and 23. Figure 24 is an example of a 'typical' monosporous culture of *C. striatus*.

None of the variations described above for *C. helenae* were observed in any of the haploid cultures of either strain of *C. striatus*[1]. No sectoring or other type of saltation occurred in any diploid cultures that were obtained in the course of this investigation.

Bacteristatic Activity of *C. helenae* and Related Species

Antagonism to a bacterial contaminant exhibited by a young haploid culture of *C. helenae* No. 1500 (Culture No. 30) was observed, and this prompted a preliminary investigation into the production of a bacteristatic substance (or substances) by *C. helenae* and other related species of *Cyathus*. In the initial stages of the work, a variety of unidentified bacterial contaminants (rods and spheres) were

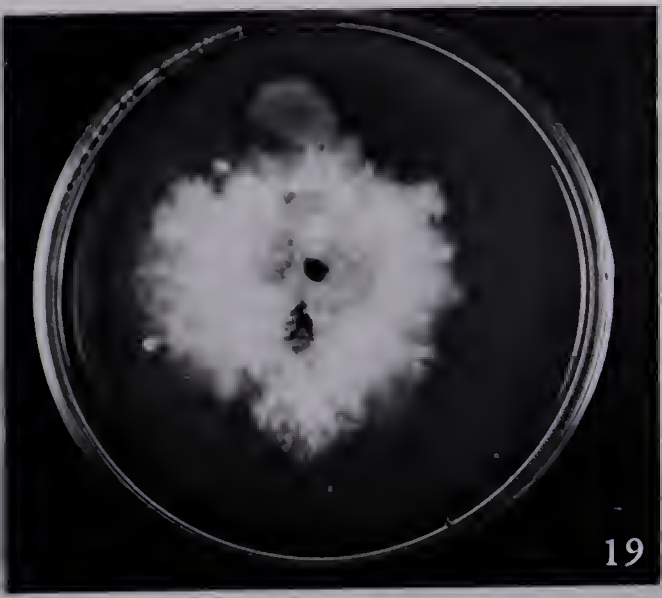
---

[1] This is a sharp line of distinction between *C. helenae* and *C. striatus* in culture. One suspects that it has a well fixed genetic basis.

PLATE IX -- Variations in Haploid Mycelia (*Cyathus helenae*)

Figs. 19 and 20.	Fast-growing saltant of much darker coloration than the parent mycelium.
Figs. 21 - 23.	Various saltants occurring at random, giving the cultures a blotchy appearance.
Fig. 24.	'Typical' monosporous culture of *C. striatus* No. 66145

Note: Figure 20 is 1.5 times actual size. All other figures are approximately 3/4 actual size.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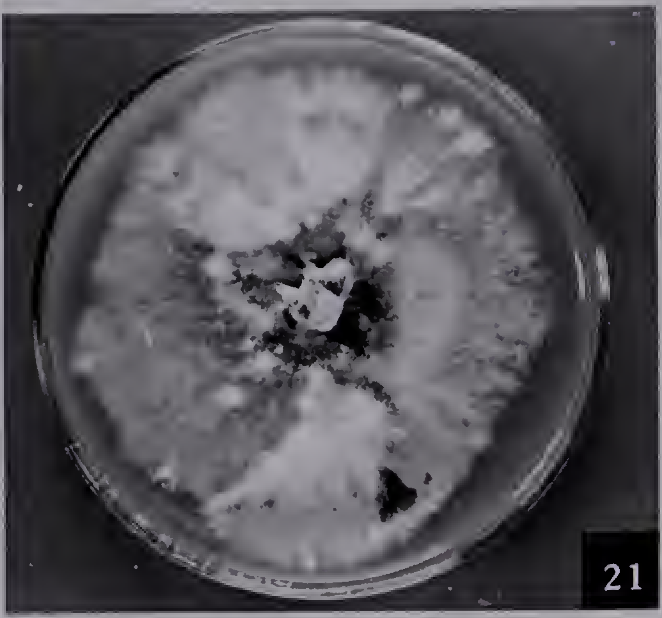
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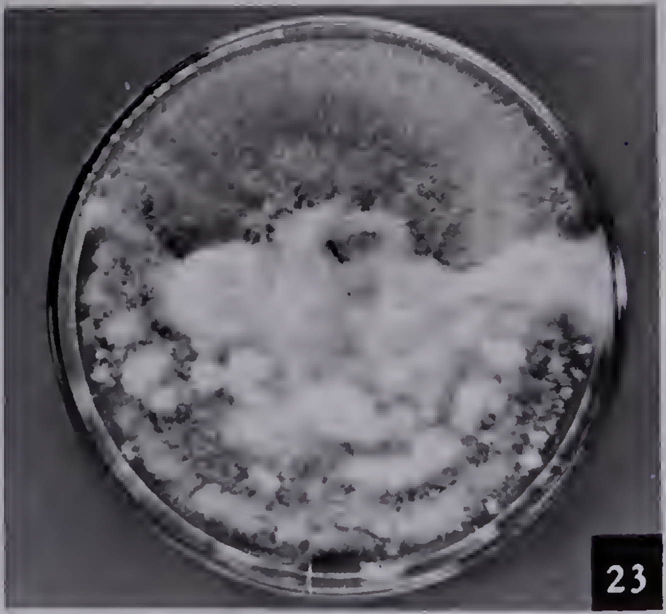
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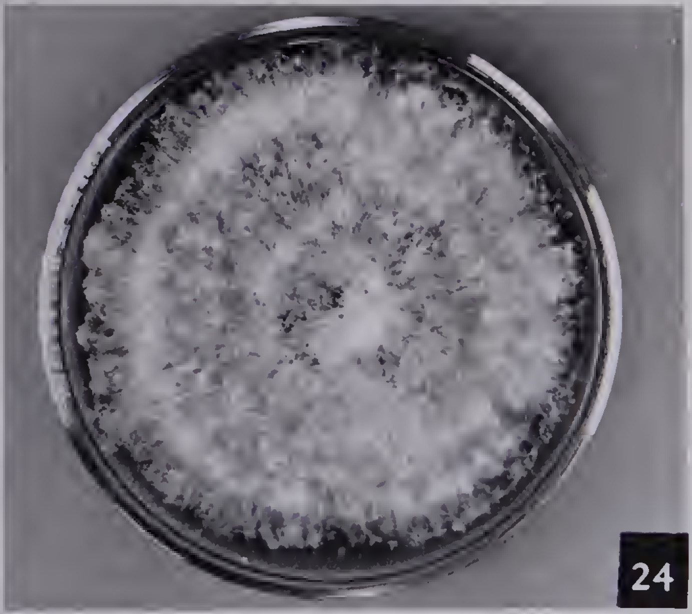
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PLATE IX

cultivated and tested for sensitivity to 'cyathin'[1]. Later, named micro-organisms were obtained for tests through the courtesy of the Departments of Microbiology and Bacteriology.

Of the named organisms tested, *Staphylococcus aureus* and all the *Micrococcus* species proved highly sensitive to the fungus product. Figures 25, 27 and 28 are photographs showing the zones of inhibition produced by cyathin against various bacteria. *Bacillus subtilis* was not inhibited by *Cyathus helenae* grown on 'normal' Brodie Medium Agar. However, when blocks prepared from a diploid culture of *C. helenae* No. 67016 (grown on 5N-Brodie Medium Liquid) were placed on a Nutrient Agar plate seeded with *Bacillus subtilis*, a zone of inhibition did result (Fig. 29).

At the edge of the zone of inhibition, there was a halo of (apparently) more intense bacterial growth, which may be seen clearly in Figs. 27, 28 and 29. After varying lengths of time (3-9 days), depending upon the diameter of the zone of inhibition, there was a resumption of some bacterial growth within the zone (Zone A, Figs. 28 and 29). In the zone containing blocks of living mycelium (Zone B, Figs. 27 and 28) there was no resumption of growth of the bacteria.

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[1] Pending further investigation, this name has been adopted to refer to the substance or substances produced by *C. helenae*, *C. striatus* and other species of *Cyathus*.

PLATE X -- Bacteristatic Activity of *Cyathus helenae*

Fig. 25. Zone of inhibition of growth of *Staphylococcus aureus* caused by *Cyathus helenae* (9/10 actual size).

Fig. 26.
- A. Stimulation of growth of *Serratia* near agar block prepared from filtrate of *Cyathus helenae*, the latter grown on 5-Normal Brodie Medium Liquid.
- B. Block of living mycelium of *C. helenae* on 1-Normal Brodie Medium Agar.
- C. Control block of 1-Normal Brodie Medium Agar (2 times actual size).

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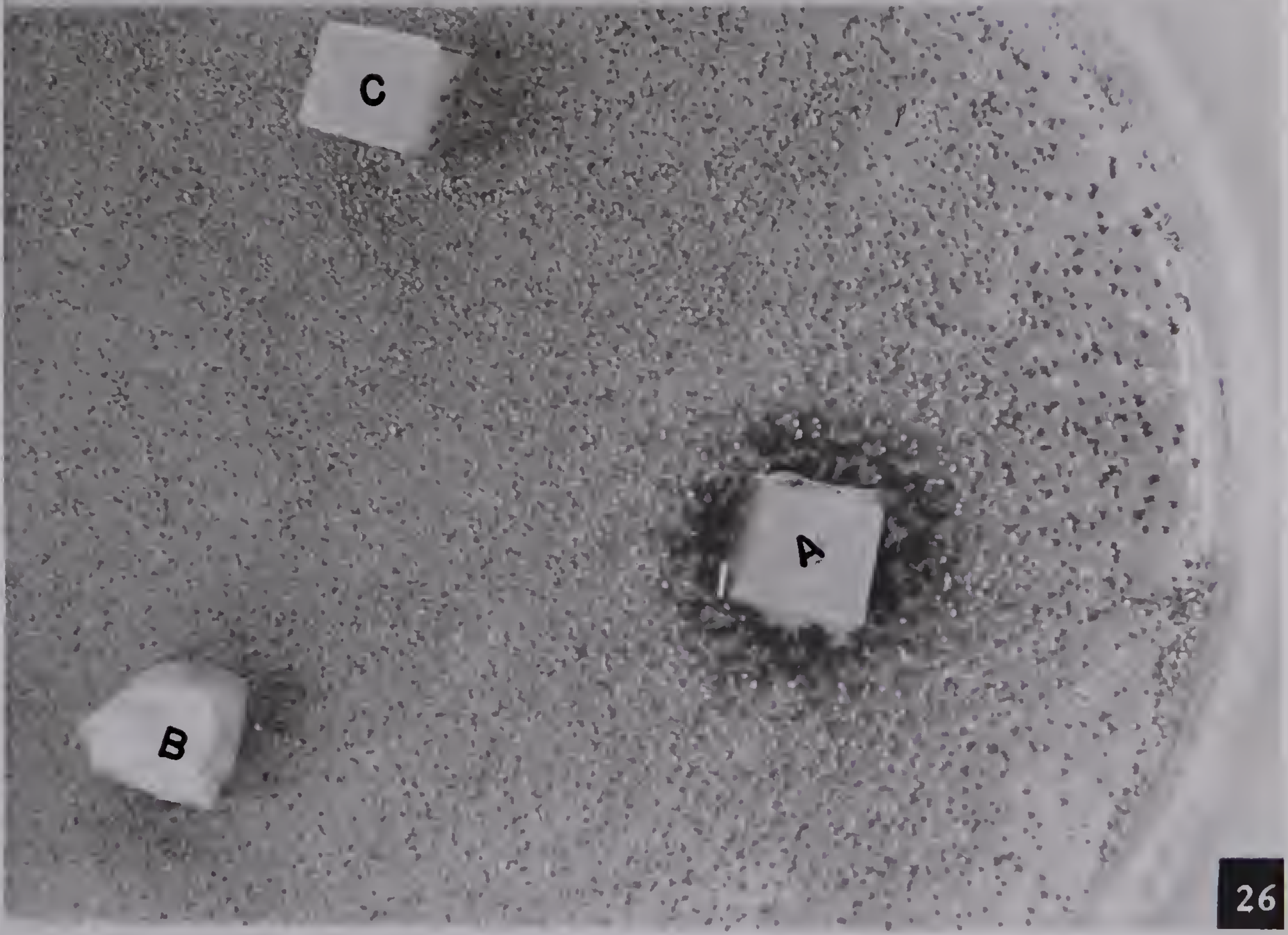

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PLATE X

PLATE XI -- Bacteristatic Action of 'Cyathin'

Fig. 27. Blocks A, B and C on a Nutrient Agar plate of *Micrococcus varians*.

Fig. 28. Blocks A, B and C on a Nutrient Agar plate of *M. flavis*. Note 'resumption' of growth in the zone of inhibition produced by block A.

Fig. 29. Inhibition of growth in *Bacillus subtilis* produced by block A. Note the narrow zone of inhibition, the intense halo at the edge of the zone, and a 'resumption' of growth within the zone in which growth had been previously inhibited.

Fig. 30. Blocks A, B and C on a Nutrient Agar plate seeded with *Serratia*.

Note: Blocks A, B and C referred to in the above figures are identical to those described relative to Fig. 26. All figures are approximately 3/4 actual size.

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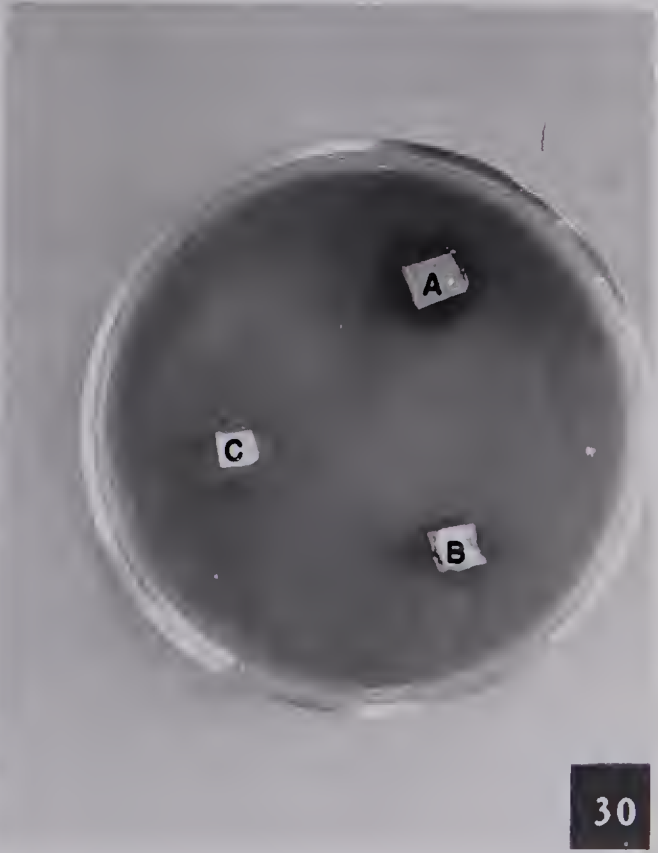

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PLATE XI

Where inhibition of growth of bacteria did not occur, there was observed a more intense growth of bacteria near the block which presumably contained the highest concentration of cyathin. A pigmented species of *Serratia* is used to demonstrate this in Figs. 26 and 30.

Several other species of *Cyathus* were tested for activity against *Staphylococcus aureus* : *Cyathus striatus*, *C. limbatus* Tul., and *C. poeppigii* Tul. produced zones of inhibition; *C. pallidus* Berk. et Curt., *C. bulleri* Brodie, *C. berkleyanus* (Tul.) Lloyd, and *C. stercoreus* (Schw.) De Toni did not produce such zones. In all these tests, blocks cut from fungal cultures as they grew on BMA plates were used.

The bacteristatic substance (or substances), produced by *Cyathus helenae* and other species tested, retained its activity (apparently undiminished) after being autoclaved for 10 minutes at 120°C.

DISCUSSION

Mating Reactions

The pattern of interfertility between haploid mycelia of *Cyathus helenae* and *C. striatus*, as reported above, can be explained on the basis of the existence of multiple alleles at either of the incompatibility loci. For many fungi, a multiple series at each locus is known [Raper (18)]. Among the Nidulariaceae, nine incompatibility factors have been reported by Fries (11) for *C. striatus*. According to Whitehouse (22) a multiple allelic series of the order of ten at each incompatibility locus is indicated by the data available for *C. striatus*. A similar pattern of interfertility -- between two distinct forms of *C. olla* -- has been reported by Brodie (5).

In the light of previous investigations, the results of the present work may now be analyzed as to the possibility of postulating the genotypes of the various haploid mycelia with regard to incompatibility factors. The data from Tables IV, V and VI are summarized on Plate XII and the postulated genotypes of the respective cultures are presented.

For purposes of comparison, *C. striatus* (No. 66145) is used herein as a 'reference' species and cultures S_6, S_4, S_7 and S_{13} have been arbitrarily designated AB, ab, aB, and Ab respectively; the latter designations are based

PLATE XII -- Compatibility Relationships Between *Cyathus helenae* and Two Strains of *C. striatus*

Relationships, based on pairing reactions, between *C. helenae* No. 1500 (H_{30}, H_{31}, etc.), *C. striatus* No. 66145 (S_6, S_7, etc.) and *C. striatus* No. 66146 (T_{10}, T_{13}, etc.). Double-headed arrows between mating types indicate sexual compatibility as evidenced by formation of clamp-connections.

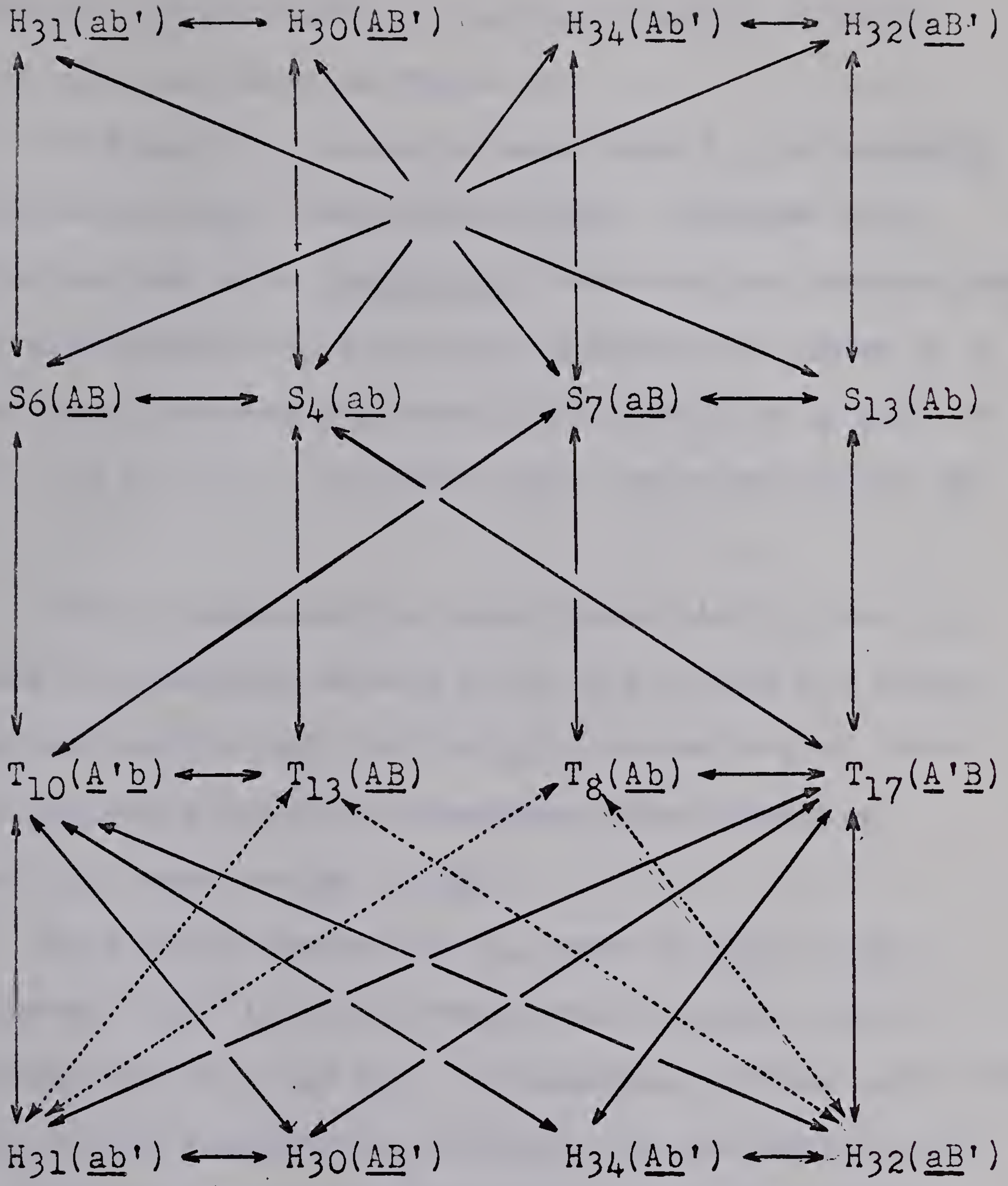
H31(ab')
H30(AB')
H34(Ab')
H32(aB')
S6(AB)
S4(ab)
S7(aB)
S13(Ab)
T10(A'b)
T13(AB)
T8(Ab)
T17(A'B)
H31(ab')
H30(AB')
H34(Ab')
H32(aB')

PLATE XII

on the sexual compatibility or incompatibility of monosporous mycelia as shown in Table II.

In Table IV, it may be seen that H_{31} is sexually compatible with S_6(*AB*) and with S_{13}(*Ab*). Because only those mycelia that give *completely* heterozygous combinations are sexually compatible, H_{31} cannot possess the genes *A*, *B* or *b*. It could possess the gene *a* and either of a pair of alleles *B*' or *b*'; i.e., it could have the constitution *aB*' or *ab*'.

If H_{31} possessed any gene other than *a* (e.g., *a*' or *A*') and the genotype were *a*'*B*' or *a*'*b*', then H_{31} would be compatible with S_4(*ab*) and S_7(*aB*) because any of these combinations would result in complete heterozygosity. Therefore, H_{31} must be *aB*' or *ab*'.

By similar reasoning, H_{30} must be *AB*' or *Ab*' because (from Table I) it is compatible with H_{31} but is incompatible with H_{34} and H_{32}. Proceeding further with this reasoning, it is possible to conclude, on the basis of the results in Table I and Table IV, that *C. helenae* mating types *AB*, *ab*, *Ab* and *aB* should be redesignated *AB*', *ab*', *Ab*' and *aB*', respectively (see Plate XII).

The results of pairing haploid mycelia of *C. striatus* (No. 66145) with the *tall* variety (*C. striatus* No. 66146) (Table V) indicate that T_{13} and T_8 possess the *same* compatibility factors as do S_6 and S_{13}; i.e., *AB* and *Ab*, respectively. T_{10} and T_{17}, however, are each compatible

with two mating types of *C. striatus* No. 66145; i.e., with S_6 and S_7, and with S_4 and S_{13}, respectively. By applying the sort of reasoning used above to determine the genotypes of the *C. helenae* cultures, it may be concluded that T_{10} is $\underline{A}'\underline{b}$ and T_{17} is $\underline{A}'\underline{B}$.

Confirmation of the plausibility of the above deductions is provided by the results of pairing haploid cultures of *C. helenae* with those of the tall variety of *C. striatus*. The results of such pairings (Table VI) are in accordance with those expected, if previous deductions are correct. A summary of interactions between the various mycelia is presented in Plate XII.

The interactions between haploid mycelia of *C. helenae* and *C. striatus* may suggest, at first, that the former is but a variety of *C. striatus*. It must be remembered, however, that *C. helenae* was recognized as a distinct species on the basis of clear-cut morphological characteristics and ecological 'preferences'. "Species should be defined", as Davis and Heywood (8) have put it, "on the basis of the variation found in nature and with due regard to their morphological discontinuity. Despite the undoubted importance of intersterility in the evolution of species, crossability criteria must be assessed, for purposes of a general classification, like any other taxonomic character and not be unduly weighted....It is only in cases of doubt, in which evidence from other sources conflicts, that evidence

from crossability can justifiably be used to tip the scales."

Furthermore, diploidization and formation of clamp-connections cannot be accepted as an infallible indication that hybrid fruit-bodies will result. Nor does it indicate that such fruit-bodies, if they were produced, would be fertile. For example, although Brodie (5) was able to obtain some fertile combinations between mycelia of *C. olla* and *C. olla* forma *anglicus*, fruit-bodies were produced by only two such matings and the fruit-bodies did not produce viable spores. In the absence of any hybrid fruit-bodies in the present study, the presence or absence of any internal isolating mechanism between the species *C. striatus* and *C. helenae* must remain mere speculation.

From the results of crossing experiments, it is possible, therefore, only to conclude, at present, that *C. helenae* and *C. striatus* are interrelated, but not necessarily interfertile in the sense that fruit-bodies and viable basidiospores can be produced from 'inter-species' matings.

Culture Characteristics and Variations

A large proportion of the haploid cultures of *C. helenae* differ markedly from those of *C. striatus*. One immediately-apparent difference is the generally lighter, and blotchy, pigmentation of the *C. helenae* cultures. An even more striking difference is the tendency of *C. helenae* mycelia to produce a variety of 'saltants' and 'sectors'.

Because the genetic basis for the observed variability has not been determined, it is difficult to ascertain the taxonomic significance of such variability. That is, it is not known whether the genetic basis for the variability in culture is the same as that responsible for an altered morphology and ecological preference in nature. The unusual habitat of *C. helenae* (in comparison with other species of *Cyathus*), however, suggests that its physiological behaviour may reflect a genetic constitution different from that of *C. striatus*.

Although the variation encountered in haploid cultures of *C. helenae* may provide an additional feature for characterization of the species, the variation may have far greater significance in its cytological and physiological implications.

The 'flat' variants (Figs. 5, 7, 8, 9 and 10) appear to resemble closely the 'flat' sectors reported by Papazian (15) in *Schizophyllum*. The 'flat' sectors of *S. commune* result from pairing two common-A homokaryons (e.g. $\underline{A}^1\underline{B}^1$ X $\underline{A}^1\underline{B}^2$, or $\underline{A}^2\underline{B}^2$ X $\underline{A}^2\underline{B}^1$), and the resulting heterokaryons retain the mating characteristics of both their component homokaryons. The 'flat' heterokaryons of *S. commune* are stable, but under appropriate conditions give rise to 'streak' sectors resembling the 'streak' variants in *Cyathus helenae* (Figs. 6, 11 and 12). The mycelium from the 'streak' sectors of *Schizophyllum commune* is stable and

homokaryotic, as is mycelium from 'streak' variants in *Cyathus helenae*. The two types of variation ('flat' and 'streak') in *Cyathus helenae*, however, arose from monosporous (homokaryotic) cultures and are not restricted to any specific mating type. The 'flat' variants occurred in each of the four mating types and 'streak' occurred in at least two.

Raper and San Antonio (18) have made an extensive study of the phenomenon of heterokaryotic mutagenesis in *Schizophyllum commune* and have characterized the resultant 'mutants' according to the outstanding morphological features of each: "streak", "puff", "fluff", "thin", "feather", "fir", "dwarf", and "vescicular". All these 'mutants' arose as sectors in common-A heterokaryons. The authors state that each type so far analyzed differs from the wild type by a single mutated locus, which is characteristic and distinct for each morphological type. Also, these mutants are distinct from mutants (of the same strains) induced by ultraviolet irradiation, and it has been demonstrated that the capacity for mutagenesis is transferable in the filtrate of aged heterokaryons. The report goes on to say that very few "mutants" had been found in an estimated 3000 homokaryotic cultures during three years but that "Roshal (unpub.) reports the regular occurrence of mutants of heterokaryotic types in homokaryotic cultures to which new nutrient was added at intervals over a prolonged

period".

It is of interest that many variants resembling the so-called mutants attributed to heterokaryotic mutagenesis in *S. commune* occur quite regularly in homokaryotic cultures of *Cyathus helenae.* It is quite conceivable that further study of the variants of *C. helenae* could yield information fundamental to understanding the pairing reactions observed in tetrapolar Basidiomycetes.

Equally intriguing is the occurrence of 'abnormally' divergent sectors (Figs. 14, 15 and 17). The term 'abnormal' is used to distinguish these sectors from the 'normal' types discussed by Pontecorvo and Gemmel (16).

Pontecorvo and Gemmel showed that the geometrical shape of a sector depends on the following three features of the changed hypha: a) its relative linear growth rate as compared with the parental hyphae; b) its positional advantage as compared with the neighboring hyphae at the time the change occurred; and c) its response to the changing environmental conditions during subsequent growth in culture. Generally, a changed hypha having a growth rate less than that of the parent can only be detected if it had a positional advantage at the time when the change occurred. However, it would sooner or later be surrounded by the parent hyphae, and further growth would be prevented. If, on the other hand, the growth rate of the changed hypha is higher than that of the parent, a sector is produced

whose radial boundaries are portions of equi-angular spirals and whose tangential boundary extends farther than the circumference of the parent culture. The distance from the origin of the sector to the center of the tangential boundary is equal in length to the curved boundaries on either side. The existence of straight radial boundaries in a sector indicates that the growth rates of the sector and the parent culture are equal, and so a true geometrical sector is formed (see Fig. 13).

Figure 15 shows an abnormal sector the straight sides of which would indicate that the growth rate of the saltant is equal to that of the parent. The advancing tangential boundary of this sector, however, has progressed very little from the point of origin. There appears to be an interaction between the saltant and the parent in the region of contact which stimulates the saltant to maintain a growth rate equal to that of the parent and prevent "invasion" of the sector by the parental mycelium. Alternatively, the parental hyphae may be converted to the sectorial type. The change is an irreversible one as evidenced by the poor growth exhibited by the saltant when it is subcultured (Fig. 16).

Figures 14 and 17 show another type of abnormal sector the geometry of which cannot be explained on the basis of growth rates alone. These sectors differ from the previously described one in that they do not appear to

originate at a single point on the circumference of the parent culture, but rather, over varying portions of it (compare apices of sectors in Figs. 15 and 17 as indicated by arrows). On being subcultured, these "C" type sectors reverted to the parental type, indicating that a mutation had not occurred, but that perhaps their formation was induced by a changed environment in the medium.

The "D" type sector shown in Fig. 15 most closely resembles the C-type reported by Isaac (13) in *Alternaria tenuis* Auct. In the latter, however, the rate of tangential spread was much greater, resulting in pronounced curved boundaries. Isaac proposed a hypothesis to explain the 'infectious' nature of the sector, suggesting that a local change in the environment might stimulate the production of a compound or a substance that would stabilize the environmental change, and if the changed condition caused a form of growth distinct from that of the hyphal tips, then a sector would be produced. Further, if the stimulus did not spread externally to the hyphae through the medium, but internally by hyphal anastamosis, then it would not be distinguishable from the effect that might be expected of an internal parasite such as a virus. An explanation for the "D" type sector in *Cyathus helenae* has not been attempted, although its persistent occurrence in one culture makes it an attractive subject for further study.

It has been mentioned above that variations

similar to those observed in *Cyathus helenae* have been attributed, in other fungi, to common-A heterokaryons. The "barrage" phenomenon [Brodie (3), Raper (18)] and formation of false clamp connections [Fulton (12)] are generally regarded as phenomena associated with the incompatible pairing of two mycelia having common B factors. In the present experiments, "barrage" has been observed in a compatible pairing in *C. striatus* (aB X Ab, Plate V) and false clamp connections were observed in a variant arising from a monosporous culture of *C. helenae* (Figs. 19 and 20). These observations are difficult to interpret in the light of currently held concepts.

Further the role of the B-factor has been considerably speculated upon [Snider (19)] in determining nuclear migration. Inadequate representation of certain mating types does not permit any conclusions to be drawn from the results of the present experiments. It is of interest, however, that cultures H_{30} and S_{13}, which act as donors of nuclei in all cases, do not themselves become diploidized when paired together, but rather, a diploid mycelium results at the region of contact between the two. Also of interest is the preponderance of ab and aB mating types relative to the AB and aB mating types in *C. helenae* (Table I). The possibility of a lethal factor linked with the A incompatibility factor cannot be discounted, since a similar situation prevails in *C. striatus* (Table

II). Alternatively, a factor stimulating germination may be associated with the b' and b factors in *C. helenae* and *C. striatus*, respectively (see revised genotypes on Plate XII).

Although cytological and physiological investigations have not been within the scope of the present study, some of the apparent irregularities of behaviour of *C. helenae* mycelia reported here seem to warrant such study.

Bacteristatic Activity

Production of bacteristatic substances by fungi has been extensively investigated. However, few studies have been conducted in this connection on the Bird's Nest Fungi [see Broadbent (2)]. Wilkins (20) reported bacteristatic activity in *Cyathus striatus* and in *Crucibulum laeve* (Bull. ex DC.) Kambly. However, Wilkins' report merely indicated the presence of bacteristatic activity in these fungi during the course of a test of hundreds of species of Basidiomycetes. No attempt was made to characterize these substances chemically.

The results of the present experiments differ somewhat from those reported by Wilkins who used *Staphylococcus aureus* and *Bacterium coli* (*Escherichia coli*) as test organisms and reported activity against both by *Cyathus striatus*. In the present studies, no inhibition of growth

of *Escherichia coli* was observed. It is possible that the medium used by Wilkins may have been more conducive to the production of bacteristatic substances than were the media used in the present work. It is also possible that different substances may have been produced under different conditions.

An interesting characteristic of cyathin is that it appears to act as a bacterial growth stimulant at low concentrations and as a growth inhibitor at higher concentrations. Also, different microbial species react differently to different concentrations of cyathin. Thus concentrations which inhibit growth of *Micrococci* (Figs. 27 and 28) stimulate growth in *Serratia* sp. (Figs. 26 and 30).

The effects of cyathin, as described above, are similar to the effects of certain inorganic ions such as copper or silver. Such ions function as enzyme activators at low concentrations and as inhibitors at higher concentrations. The effects of cyathin, however, appear to differ from those of an inorganic ion in that cyathin stimulates growth of all micro-organisms tested, indicating that its effects are more general among the microbial species than are those of an inorganic ion. It is also possible, that the apparent stimulation of growth in species where growth is not inhibited (e.g. *Serratia* sp. Fig. 26) is due to the utilization of metabolites produced by *Cyathus helenae*, rather than a direct stimulatory effect of such metabolites.

Thermal stability of cyathin in aqueous solution,

reported above under Results and Observations, leads one to suspect that the cyathin molecule may be relatively small. However, only extensive work designed to lead to the purification of cyathin will reveal the true nature and structure of this interesting metabolite.

The simple investigation into the production of bacteristatic substances by *C. helenae* and *C. striatus* reported herein may, however, have value in suggesting that the two species may be closely related in certain aspects of their metabolism. Of greater interest, however, may be the significance of the products of their metabolism to microbial physiology. It is the writer's opinion that much fruitful information can be obtained from further investigations of the physiology of *C. helenae* and other species of *Cyathus*, especially with regard to the metabolites discussed above.

SUMMARY

1. Basidiospores of *Cyathus helenae* germinate at 22°C on a dilute Sucrose Peptone Agar, following 48 hours incubation at 37°C.

2. *C. helenae* is heterothallic and tetrapolar, as are most other species of the Nidulariaceae.

3. The interactions of haploid mycelia of *C. helenae* and *C. striatus* suggest genetic relationship between the two species.

4. Although the unusual (and probably unique) habitat of *C. helenae* provides the species with an effective external isolating mechanism, the results of crossing experiments do not conclusively demonstrate the presence of an internal mechanism which would prevent hybridization between *C. helenae* and *C. striatus*.

5. Haploid cultures of *C. helenae* differ markedly from those of *C. striatus*. The most striking difference is the tendency of a high proportion of *C. helenae* cultures to produce a variety of saltants and sectors which do not appear in mycelia of *C. striatus*.

6. Many variants of monosporous cultures of *C. helenae* resemble variants known to be associated with heterokaryotic mycelia in *Schizophyllum commune* and other tetrapolar species of Basidiomycetes.

7. Both haploid and diploid cultures of *Cyathus helenae* are able to metabolize heat-stable substances which act as bacterial growth stimulants at low concentrations, and as growth inhibitors at high concentrations. Substances with similar properties are metabolized by some of the other species of *Cyathus*.

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